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Activity of purified biosynthetic proteinase of human immunodeficiency virus on natural substrates and synthetic peptides

(polyprotein processing/peptide cleavage/acquired immunodeficiency syndrome)

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ABSTRACT **Retroviral capsid proteins and replication enzymes are synthesized as polyproteins that are proteolytically processed to the mature products by a virus-encoded proteinase. We have purified the proteinase of human immunodeficiency virus (HIV), expressed in *Escherichia coli*, to ≈90% purity. The purified enzyme at a concentration of ≈20 nM gave rapid, efficient, and specific cleavage of an *in vitro* synthesized *gag* precursor protein. Purified HIV proteinase also induced specific cleavage of five decapeptide substrates whose amino acid sequences corresponded to cleavage sites in the HIV polyprotein but not of a peptide corresponding to a cleavage site in another retrovirus. Competition experiments with different peptides allowed a ranking of cleavage sites. Inhibition studies indicated that the HIV proteinase was inhibited by pepstatin A with an IC_{50} of 0.7 μM.**

The capsid and nonstructural proteins of all retroviruses, including human immunodeficiency virus (HIV), are synthesized as polyprotein precursors that are proteolytically processed to the mature viral proteins by a virus-encoded, virus-associated proteinase (for a review see ref. 1). Viral proteinases (PR; for the new nomenclature for common retroviral proteins see ref. 2) have been purified from virions and biochemically characterized for a number of avian (3, 4) and mammalian (5–7) retroviruses. These enzymes share limited amino acid sequence homology with members of the aspartic proteinases (8, 9) and invariably contain the sequence Asp-Thr(Ser)-Gly corresponding to the catalytic center of cellular aspartic proteinases. However, retroviral enzymes are much smaller than cellular aspartic proteinases and contain only a single homologous catalytic center.

The proteolytic activity of HIV has been mapped to a 11-kDa protein that is encoded immediately upstream of the viral reverse transcriptase (RT) and appears to be generated by autocatalytic release from a larger precursor (10–12). Replication of infectious HIV particles is entirely dependent on the generation of active PR, and a mutation of the putative active site Asp residue in the PR gene resulted in the production of "immature," noninfectious particles consisting of uncleaved precursor proteins (13). Although genetic and biochemical evidence has mapped the proteolytic activity to this specific segment of the HIV genome, the mechanisms of PR formation and action are still unknown. Is dimerization of the enzyme a prerequisite for function (14) and, if so, how are the initial cleavages performed? Does the enzyme require activation or relief of inactivation or is processing confined to a specific (virus-induced?) compartment (for a review see ref. 1)? In addition to providing insight into the biology of retrovirus replication, studies of the enzymatic functions of HIV have recently received much attention as these enzymes are potential targets for antiviral drugs. HIV PR, like the proteinases of other human pathogenic viruses, such as poliovirus, appears sufficiently distinct from cellular endopeptidases that inhibitors of the viral enzyme may not be harmful to the host. These studies require sufficient quantities of pure, active enzyme and functional assays in a peptide-based system as well as on a natural substrate.

We report here purification of enzymatically active HIV PR from an *Escherichia coli* expression system. The purified enzyme can cleave its natural substrate, obtained by translation *in vitro*, as well as five different decapeptides designed according to cleavage sites in the HIV polyprotein. Processing of these peptides occurred specifically at the site that is also cleaved *in vivo*, and a non-HIV-specific peptide was not cleaved by HIV PR. Competition experiments with different peptide substrates allowed ranking of the peptides according to the relative rates of cleavage.

MATERIALS AND METHODS

Purification of HIV PR. Construction of plasmid pHIVproPII and expression in *E. coli* BL21(DE3) has been described (12). In the present study we used BL21(DE3) containing the T7 lysozyme gene on a chloramphenicol-resistance plasmid [BL21(DE3)plysS] as the expression strain. T7 lysozyme has been shown to inhibit T7 RNA polymerase (15), thus providing a more stringent control of base line expression. Bacteria transformed with pHIVproPII were grown in M9CA medium (16) with ampicillin and chloramphenicol in a 14-liter fermentor (Microferm MMF-14, New Brunswick Scientific, New Brunswick, NJ), essentially as described (17). Bacteria were collected by centrifugation and stored as a wet paste at −80°C.

For each purification, bacterial paste was thawed in 50 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes), pH 6.5/0.1 M NaCl/10 mM $MgCl_2$/1 mM EDTA and lysed in a French pressure cell at 75 MPa. The lysate was centrifuged for 15 min at 10,000 × *g* and 60 min at 200,000 × *g*, the supernatant was collected and made 10 mM in EDTA, and $(NH_4)_2SO_4$ was added to 50% saturation. The precipitate was redissolved in 50 mM Tris·HCl, pH 8.0/30 mM NaCl/1 mM EDTA and layered on a DEAE-cellulose column (Whatman DE52) equilibrated with the same buffer. The flow-through was adjusted to pH 6.5, made 1 M in $(NH_4)_2SO_4$, and layered on a hexylagarose (Sigma) column equilibrated with 1 M $(NH_4)_2SO_4$ in 50 mM Mes, pH 6.5/1 mM EDTA (buffer A).

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Abbreviations: HIV, human immunodeficiency virus; TFA, trifluoroacetic acid; PR, protease; RT, reverse transcriptase.

The column was washed with the same buffer and with 0.85 M $(NH_4)_2SO_4$ in buffer A and was eluted with 50 mM $(NH_4)_2SO_4$ in buffer A. Protein was precipitated with $(NH_4)_2SO_4$, redissolved in 50 mM Mes, pH 6.5/150 mM NaCl/1 mM EDTA (buffer B), and loaded on a 60 × 1.6 cm column of Sephadex G-50 fine (Pharmacia) in buffer B at 9 ml/hr. Peak fractions were precipitated with $(NH_4)_2SO_4$, redissolved in buffer B, and loaded on a Superose 12 HR10/30 column (Pharmacia) at 0.5 ml/min.

In a control experiment BL21(DE3)plysS were transformed with plasmid pHIVproP (12). Purification was performed as described above except that the final Superose 12 and the hexylagarose step were omitted. Instead, the DEAE flow-through was precipitated by addition of $(NH_4)_2SO_4$ and the precipitate was redissolved in buffer B and loaded on the Sephadex G-50 column.

***In Vitro* Transcription and Translation.** Synthetic RNAs were transcribed with T7 RNA polymerase *in vitro* (18) from plasmids pHIVg/pII and pHIV FSII (12). Both plasmids contain the HIV cDNA sequence from nucleotides 221 to 2129 but pHIV FSII has a 4-base-pair insertion at nucleotide 1640 of the HIV cDNA leading to a switch from the *gag* reading frame to the *pol* reading frame (12). Synthetic RNAs (final concentration, 50 μg/ml) were translated in rabbit reticulocyte lysate (Promega Biotec) for 60 min at 30°C as described by the supplier.

Synthesis and Purification of Peptides. Peptides were synthesized by the Merrifield method on an Advanced Chemtech automated synthesizer and were cleaved from the resin by liquid HF at 0°C in the presence of anisole and dimethyl sulfide as scavengers. Dried peptide resin mixtures were washed with diethylether and peptides were extracted with water and 80% acetonitrile in water. The extracts were lyophilized and the crude peptides were purified by reversed-phase HPLC using 0.1% aqueous trifluoroacetic acid (TFA)/acetonitrile-based mobile phases. The lyophilized products were characterized by fast atom bombardment mass spectrometry, HPLC, and amino acid analysis.

Peptide Cleavage by HIV PR. Reactions were performed at 30°C in 20 or 40 μl of 50 mM sodium phosphate, pH 6.0/25 mM NaCl/5 mM EDTA/1 mM dithiothreitol with 0.44 mM decapeptide as substrate and 2 μl of partially purified PR (in purification buffer B). The reaction was quenched with a 4-fold excess of 0.1% TFA and frozen. Reaction products were analyzed by reversed-phase HPLC using a Vydac C_{18} analytical column with a gradient from 95% A/5% B to 100% B (A is 0.1% TFA in water; B is 0.09% TFA in 60% acetonitrile/40% water) in 30 min at a flow rate of 1 ml/min. Absorbance was monitored at 215 nm. Cleavage products were identified by sequencing on an Applied Biosystems model 477A sequencer equipped with a model 120A phenylthiohydantoin analyzer. Fast atom bombardment mass spectrometry was carried out on a Kratos MS-80 RFAQ double-focusing mass spectrometer.

Competition Experiments. Reactions were carried out at 37°C in buffer consisting of 50 mM Mes, 25 mM NaCl, and 5% dimethyl sulfoxide at pH 6.0. When the substrate contained methionine or cysteine residues, 1 mM dithiothreitol was included. Substrate concentration was 250 μM BI-P-136 and 250 μM another decapeptide. Reactions were started by adding 5–10 μl of enzyme in purification buffer B. For each time point a 20-μl sample was removed and added to 0.1 ml of 2% TFA in water. Peptides were separated on a Nucleosil C_{18} column with a 26-min linear gradient at 1 ml/min from 9% to 56% acetonitrile in water with 0.05% TFA and elution was monitored at 210 nm. For each substrate and its products, the area under the combined peaks was independent of extent conversion of the substrate. Thus, substrate and products were detected and recovered with equal efficiencies and comparison of areas yielded extent conversion.

When two substrates compete for the active site of an enzyme, kinetic analysis yields the ratio of V_{max}/K_m (19) rather than comparisons of either V_{max} or K_m individually. The rate constant ratios were determined from the extent of conversion of each substrate using the equation $(V_{max}/K_m)_1/(V_{max}/K_m)_2 = \log(1 - F_1)/\log(1 - F_2)$, where F is the fraction of substrate that is converted to products (20).

RESULTS

Purification of Biosynthetic HIV PR. We (12) and others (10, 11, 13, 21, 22) have recently reported expression of active HIV PR in *E. coli*. In our initial studies (12) we demonstrated that HIV PR was produced in *E. coli* BL21(DE3) transformed with the expression plasmid pHIVproPII, which contains the 5′ terminal part of the *pol* reading frame of HIV under the control of upstream elements of bacteriophage T7 gene *10*. The mature enzyme (99 amino acids; refs. 10 and 22) was released by autocatalytic cleavage of the precursor (12). The expression level for HIV PR was low and the protein could not be identified on a Coomassie blue-stained gel of bacterial lysates (Fig. 1*a*, lane 1). Large quantities of bacterial cells were grown in a bench-top fermentor because of this low-expression level.

We followed purification of the enzyme by immunoblot analysis using a polyclonal antiserum against HIV PR. This antiserum detected a single protein in lysates of induced bacteria (Fig. 1*b*, lane 3) and did not react with lysates that did not carry the expression plasmid (Fig. 1*b*, lane 1). The majority of HIV PR was soluble after high-speed centrifugation (Fig. 1*a*, lanes 2 and 3). The enzyme was quantitatively precipitated by $(NH_4)_2SO_4$ at 50% saturation (Fig. 1*a*, lane 4) and passed through a DEAE-cellulose column at low ionic strength. After this step, the enzyme could be visualized on a stained gel (Fig. 1*a*, lane 5) and was determined by laser densitometry to be ≈1% of total protein. The material was

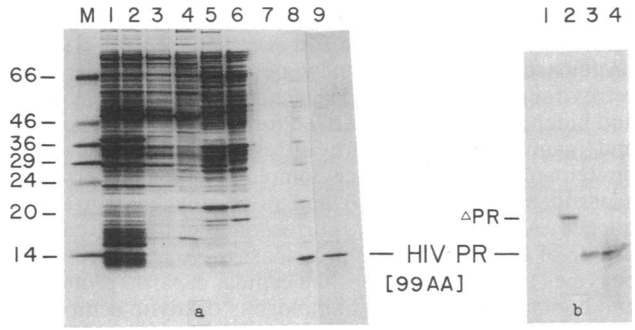

FIG. 1. Purification of HIV PR. (*a*) Aliquots taken at each step of the purification protocol were separated by NaDodSO$_4$/10–20% polyacrylamide gel electrophoresis and proteins were stained with Coomassie blue. AA, amino acids. Lane M refers to a marker lane containing 1 μg each of seven molecular size standards (indicated in kDa). In lanes 1–6, 20 μg of protein (determined using the Bio-Rad assay) was loaded, whereas lanes 7–9 contained ≈0.5 μg, ≈5 μg, and ≈1.5 μg, respectively. Lanes: 1, lysate of induced bacteria carrying pHIVproPII; 2, S-10 supernatant; 3, S-200 supernatant; 4, 50% $(NH_4)_2SO_4$ precipitate; 5, DEAE flow-through; 6, hexylagarose eluate; 7, pooled peak fractions from G-50 column; 8, $(NH_4)_2SO_4$ precipitate of G-50 pool; 9, pooled peak fractions from Superose 12 column. (*b*) Immunoblot analysis of bacterial fractions. Twenty micrograms of lysate from bacteria carrying the vector plasmid (lane 1), the deletion plasmid pHIVproP (lane 2), or the expression plasmid pHIVproPII (lane 3) and 1.5 μg of purified HIV PR (lane 4) were analyzed by gel electrophoresis and proteins were transferred to nitrocellulose for 2 hr at 0.2 A. The nitrocellulose paper was then probed with a polyclonal antiserum against HIV PR. Detection was with goat anti-IgG (rabbit), coupled to alkaline phosphatase (Tago), and with indolyl phosphate/nitro blue tetrazolium as the substrate/indicator system, essentially as described (23).

then passed over a hexylagarose column (Fig. 1*a*, lane 6) followed by molecular size chromatography on a Sepharose G-50 column (Fig. 1*a*, lanes 7 and 8) and a Superose 12 column. After this multistep purification, HIV PR was ≈90% pure as determined by laser densitometry of the gel shown in Fig. 1*a*, lane 9. Since the initial expression was low it is difficult to assess the level of overall recovery of protein but we could isolate ≈25 μg of HIV PR from 10 g of wet *E. coli* cell paste. Purified HIV PR was stable at −80°C for several weeks and did not appreciably lose activity on repeated freeze-thaw cycles.

HIV PR obtained by this purification reacted strongly with the polyclonal antiserum in immunoblot analysis (Fig. 1*b*, lane 4). The N-terminal sequence of the protein was determined by automated Edman degradation on an Applied Biosystems model 477A protein sequencer, using an *o*-phthaldialdehyde cycle in the first step to block N-terminal residues present other than proline as described by the supplier. Sequencing of the first five residues yielded Pro-Gln-Ile-Thr-Leu, the sequence found at the amino terminus of HIV PR (10, 22).

Cleavage of HIV *gag* Precursor by Purified HIV PR. In our initial experiments we observed that crude extracts from bacteria carrying plasmid pHIVproPII induced cleavage of *in vitro* synthesized *gag* precursor pr53, whereas bacterial extracts carrying pHIVproP, which has a deletion of the 17 C-terminal codons of HIV PR, did not catalyze this reaction (12). We now used this trans assay to monitor activity of purified HIV PR.

Fig. 2, lane 4, shows the products of translation of a synthetic mRNA containing the coding region for HIV *gag* and, in a different reading frame, for PR. The primary translation product was the *gag* precursor pr53, which was completely stable to incubation in buffer alone (Fig. 2, lane 5; see also ref. 12). Incubation of the *gag* precursor with purified HIV PR (Fig. 2, lanes 7–13) gave rapid and efficient processing to HIV capsid proteins. In lane 7, ≈0.5 μM HIV PR was present in the incubation and in lane 8, ≈20 nM

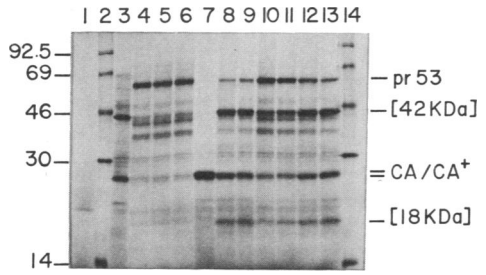

FIG. 2. Proteolytic processing of *in vitro* synthesized HIV *gag* precursor proteins. Samples of translation mixtures programed with no mRNA (lane 1), FSII RNA (lane 3), or g/pII RNA (lanes 4–13) either were mixed directly with sample buffer (lanes 1–3) or incubated with or without addition of bacterial fractions. Cleavage reactions were carried out in 20 μl final volume using 1.5 μl of translation mix as substrate and 1 μl of purified enzyme. Incubation was in 50 mM Mes, pH 6.0/20 mM NaCl/5 mM EDTA for 60 min at 30°C unless otherwise stated. Reactions were analyzed on 12.5% polyacrylamide/$NaDodSO_4$ gels. Kodak XAR-5 film was exposed to the dried gels for 16 hr. Lanes: 1, no mRNA; 2, mixture of ^{14}C-methylated proteins obtained from Amersham (sizes indicated in kDa); 3, FSII RNA; 4, g/pII RNA, no incubation; 5–13, g/pII RNA incubated with buffer alone for 1 hr (lane 5), partially purified extract from bacteria carrying plasmid pHIVproP for 1 hr (lane 6), ≈0.5 μM HIV PR for 1 hr (lane 7), or ≈20 nM HIV PR for 1 hr at 30°C (lane 8), 1 hr at 37°C (lane 9), 2 min at 30°C (lane 10), 5 min at 30°C (lane 11), 15 min at 30°C (lane 12), and 30 min at 30°C (lane 13); 14, marker lane as in lane 2. Note that a protein of ≈42 kDa was seen even without incubation with PR (lanes 4–6). This protein is distinct from the cleavage intermediate and is derived from internal initiation at Met-142 of the *gag* reading frame.

purified HIV PR was used. The lower concentration of enzyme gave almost complete cleavage of pr53 to yield the major capsid protein CA/Ca^+ (CA^+ specifies the C-terminally extended capsid protein p25; ref. 24) and two intermediates of ≈42 kDa and ≈18 kDa. Both intermediates were completely processed when 0.5 μM HIV PR was used. The 18-kDa protein probably corresponds to the C-terminal intermediate of *gag*, which is further processed to the nucleocapsid protein NC and protein p9 (24). By using a different gel system, we could detect a product that migrates at ≈8–10 kDa (data not shown). The 42-kDa protein probably corresponds to an intermediate containing the matrix protein MA and CA/CA^+ (12). The 42-kDa intermediate and CA comigrated with the corresponding products from an *in vitro* synthesized precursor containing equimolar amounts of *gag* and PR, which yielded efficient autocatalytic processing (Fig. 2, lane 3). Incubation of pr53 with material purified from bacteria carrying the deletion plasmid pHIVproP (12) did not result in any cleavage (Fig. 2, lane 6), although 50-fold more protein was used than in the experiment shown in lane 7.

We followed processing of pr53 with purified HIV PR in a time-course experiment (Fig. 2, lanes 10–13). Cleavage occurred very rapidly and cleavage products were already observed after a 2-min incubation (Fig. 2, lane 10). The cleavage assay was optimally performed at 30°C (Fig. 2, lanes 8 and 9). The pH optimum for cleavage was between pH 5.5 and 6.5, with pH >7 strongly inhibiting the activity, and the optimal salt concentration was between 20 and 100 mM NaCl (data not shown).

Cleavage of Peptide Substrates by Purified HIV PR. While the purification protocol was being developed, we tested the activity of partially purified HIV PR on synthetic peptide substrates. These peptides were modeled according to cleavage sites in the HIV *gag-pol* precursor. Fig. 3 shows that HIV PR induced cleavage of a decapeptide containing the sequence at the PR/RT cleavage site in the HIV *gag-pol* precursor (peptide BI-P-127; see Table 1). Two products, distinct from the parent peptide, were resolved by reversed-phase HPLC (Fig. 3 *b* and *c*). Sequence analysis of the peptide products confirmed that they had the sequences C-T-L-N-F and P-I-S-P-I, respectively, and therefore resulted from cleavage at the Phe/Pro bond. No other peptide material could be found in any other fraction. Integration of

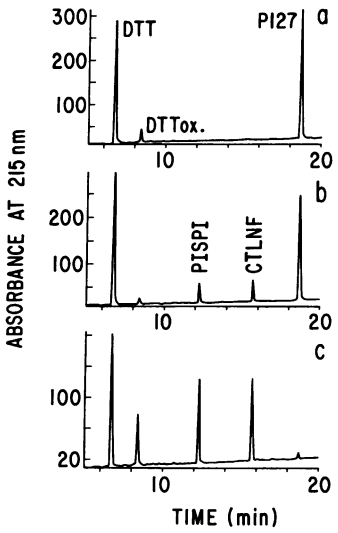

FIG. 3. Reversed-phase HPLC analysis of peptide BI-P-127 and HIV PR-generated cleavage products. The peptide was incubated for 4 hr in buffer alone (*a*) or for 1 hr (*b*) or 4 hr (*c*) with purified HIV PR. Monitoring was performed at 215 nm. DTT and DTTox. correspond to dithiothreitol and oxidized dithiothreitol, respectively. Cleavage products are identified by their amino acid sequence.

Table 1. Relative cleavage of HIV peptide substrates

Cleavage site*	Sequence					↓							Code	$(V_{max}/K_m)_{rel.}$†
	P5	P4	P3	P2	P1		P1′	P2′	P3′	P4′	P5′			
p6*/PR	V	S	F	N	F	*	P	Q	I	T	L	– NH_2	BI-P-136	1.00
CA^+/NC	T	A	T	I	M	*	M	Q	R	G	N	– NH_2	BI-P-140	0.20
MA/CA	V	S	Q	N	Y	*	P	I	V	Q	N	– NH_2	BI-P-138	0.07
CA/CA^+	K	A	R	V	L	*	A	E	A	M	S	– NH_2	BI-P-144	0.04
PR/RT	C	T	L	N	F	*	P	I	S	P	I	– NH_2	BI-P-127	0.03
RT/IN (avian)	Ac–T	F	Q	A	Y	*	P	L	R	E	A	– NH_2	BI-P-102	<0.005

IN, integrase protein.

*Cleavage sites within the HIV *gag-pol* polyprotein are designated according to the new nomenclature (2), except for the N-terminal product from the *pol* reading frame (p6*), for which there is no new name. CA^+ specifies the C-terminally extended capsid protein p25 (24).

†Relative values of V_{max}/K_m were determined by using competition experiments. Each value is an average of at least three determinations and is reproducible to ±20%.

the absorbance peaks corresponding to the parent peptide and the two cleavage products showed complete conversion of parent to product and no losses were observed. To further support the specificity of this cleavage, peptide BI-P-127 was incubated with material purified from bacteria carrying the deletion plasmid (pHIVproP), which does not contain active HIV PR. This incubation did not result in any processing of the peptide, although 50-fold more protein was used than in the experiments with active HIV PR (data not shown).

In addition to peptide BI-P-127, four other decapeptides, corresponding to cleavage sites within the HIV polyprotein, were synthesized. Peptide BI-P-136, which contains the N-terminal cleavage site of HIV PR (Table 1), was specifically cleaved at the Phe/Pro bond to release fragments corresponding to the N terminus of the proteinase and the C terminus of the upstream *pol* sequences (p6*). Both cleavage products were identified by sequence analysis of materials in the respective peaks after HPLC analysis. Fast atom bombardment mass spectrometry showed total conversion of material in a peak of 1164 atomic mass units to material of 570 atomic mass units (C-terminal fragment) and of 613 atomic mass units (N-terminal fragment) corresponding to the parent peptide and the two products after cleavage at the Phe/Pro bond, respectively. Three more decapeptides corresponding to the N- and C-terminal cleavage sites of the capsid protein CA/CA^+ (24) were synthesized. These peptides contained a Tyr/Pro (BI-P-138), Leu/Ala (BI-P-144), or Met/Met (BI-P-140) dipeptide as scissile bond. Incubation of these peptides with HIV PR gave specific cleavage in all cases (Table 1), and all products were identified by sequence analysis.

In addition to these HIV-specific peptides we studied the activity of HIV PR on a peptide that did not contain any HIV cleavage site. Peptide BI-P-102 corresponds to the cleavage site between the α-subunit of RT and IN of avian sarcoma-leukosis virus (Table 1; ref. 25) and contains a Tyr/Pro dipeptide as scissile bond but was completely stable to incubation with HIV PR under conditions where complete cleavage of the HIV-specific peptides occurred (Table 1).

In a separate set of experiments, we determined the relative susceptibility of these peptides to cleavage by HIV PR. Relative values of V_{max}/K_m for all substrates (Table 1) were obtained in experiments where two peptides, BI-P-136 and another, were incubated simultaneously with HIV PR and the substrates competed for the active site. This method requires fewer experiments than determining absolute values for V_{max}/K_m for each substrate and is not affected by variations in enzyme activity in different experiments. Competition of peptide BI-P-136 with each of the other five peptides resulted in the relative values V_{max}/K_m shown in Table 1. The best substrate, BI-P-136, was 5-fold more active than any other peptide tested, and peptide BI-P-140, containing a Met/Met dipeptide as the scissile bond, was cleaved at a significantly faster rate than the other three HIV-specific peptides.

The approximate turnover number for HIV PR was estimated from the rate of substrate turnover and an estimate of the enzyme concentration. Cleavage of 250 μM BI-P-136 was complete within 20 min in the presence of ≈200 nM HIV PR. This corresponds to a turnover number of ≈1 s^{-1}, if we assume that the enzyme was saturated under these conditions and the observed rate thus represents V_{max}. This value is a lower limit for the turnover number since the enzyme could not possibly be saturated for the entire course of cleavage of BI-P-136.

Since HIV PR was shown to be inhibited by high concentrations of pepstatin A (12, 21, 26), we determined the effect of pepstatin A on peptide cleavage (using peptide BI-P-136 as substrate) by purified HIV PR. These experiments gave an IC_{50} value for pepstatin A of 0.7 μM.

DISCUSSION

In this communication we report purification of active HIV PR to 90% purity. The enzyme was expressed in bacteria to a relatively low level but it was largely soluble and could therefore be purified by making use of its basic net charge, hydrophobicity, and low molecular mass. These results are in contrast to a report showing partial purification of HIV PR from *E. coli* (21), in which the enzyme was highly insoluble. Moreover, these authors followed purification of the enzyme only by immunoblot analysis and the degree of purity of their partially purified proteinase was not stated.

The purified enzyme was shown to be active on an *in vitro* synthesized *gag* precursor and on decapeptide substrates. Processing of the structural precursor was very efficient, giving almost complete cleavage with ≈20 nM enzyme. Thus, HIV PR was significantly more active on its natural substrate than poliovirus 3C, for which a concentration of 25 μM was required to achieve equally good cleavage of its natural substrate (17). An efficient proteolytic enzyme is desirable for HIV since *gag* precursor and PR are produced in a ratio of about 10:1 (27) and every enzyme molecule must cleave at least 50 peptide bonds to achieve complete processing of the *gag* and *gag-pol* polyproteins.

Purified HIV PR induced cleavage of five decapeptides that contained sequences corresponding to cleavage sites within the HIV polyprotein. Specific cleavage of peptides was also observed in a very recent study using chemically synthesized HIV PR (26). However, no kinetic analysis was performed in this study and the experiments were carried out on peptides of variable length and at a different pH. These differences may account for the different "ranking" of peptides these authors observed. Moreover, the chemically synthesized PR seems to be considerably less active than PR purified from *E.*

coli. This may be due to the presence of a high percentage of inactive molecules that accumulated during chemical synthesis.

On the basis of amino acid comparisons between different viruses, a consensus sequence for retroviral cleavage sites has been proposed (28), suggesting a generally hydrophobic pattern of amino acids surrounding the cleavage site with Tyr (Phe)/Pro being the most frequently occurring P1 and P1′ residues, respectively. Three of our five decapeptide substrates match this pattern of amino acids very closely, whereas the other two are dissimilar but still contain primarily hydrophobic residues surrounding the scissile bond. Additional substrate determinants are likely to reside in amino acids on both sides of the scissile bond but larger data sets are required to define these determinants. HIV PR did not cleave an oligopeptide containing a Tyr/Pro cleavage site that served as a substrate for PR of avian sarcoma-leukosis virus (25). On the other hand, avian PR could process several oligopeptides corresponding to HIV cleavage sites (25). It remains to be seen whether the substrate requirements for small peptides generally match those for cleavage of viral polyproteins. Accessibility of potential cleavage sites and structurally flexible contexts are obvious additional determinants.

Cleavage of different dipeptide bonds by one enzyme raises the possibility of differences in the relative susceptibility of these sites. This could be a regulatory feature in that processing may be required for activation or inactivation of functional domains within the polyprotein during and after assembly of the virion. In competition experiments we demonstrated that a peptide corresponding to the N-terminal cleavage site of PR was processed significantly faster than all other peptide substrates. These results suggest that this cleavage, which leads to separation of the structural proteins of the nucleocapsid from the replication enzymes, may be a very efficient first step in the processing pathway. A similar relative order of processing steps is observed in picornavirus replication where cleavage between the structural and nonstructural domains of the viral polyprotein is a very efficient first step in the processing cascade (1). Recently, it was shown that the CA/NC cleavage can occur at three different sites within a 16-amino acid sequence (24). Our results indicate that purified HIV PR can cleave at least two of these sites and that the Met/Met site may be the preferred cleavage site since processing of a peptide containing the Leu/Ala site was significantly slower.

Retroviral proteinases are believed to be aspartic proteinases and it is of interest in this regard that pepstatin A, which had been shown to inhibit polyprotein cleavage by retroviral enzymes at a very high concentration (>0.1 mM; refs. 9, 12, 21, and 26), inhibited peptide cleavage by purified HIV PR with an IC_{50} of 0.7 μM. This inhibitory effect is still very weak compared to a K_i for pepsin of 4.5×10^{-11} M (29) but is comparable to the effect of pepstatin A on renin (K_i = 0.1–1 μM; ref. 29), consistent with the classification of the retroviral enzymes as aspartic proteinases.

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